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DEVELOPMENT OF INDIGENOUS FUNGAL PATHOGENS FOR INTEGRATED MANAGEMENT OF SCENTLESS CHAMOMILE IN SASKATCHEWAN

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Development of Indigenous Fungal Pathogens for Integrated Management of Scentless Chamomile in Saskatchewan

Gary Peng and Karen L. Bailey
Bioproducts and Bioprocessing
107 Science Place, Saskatoon, SK S7N 0X2

Abstract

Scentless chamomile is a noxious weed in western Canada, with natural tolerance to many herbicides. The indigenous fungal pathogen *Colletotrichum truncatum* (Ct), with moderate virulence and host specificity on scentless chamomile, was identified as a biocontrol agent against this weed. In this project, eight studies were conducted to determine detailed infection requirements by Ct and Ct-herbicide synergy for optimal efficacy in weed control, explore leading-edge technologies for mass production and formulation of Ct spores, uncover molecular characteristics of the fungus, and evaluate the effectiveness of Ct plus herbicide formulations under field conditions. The optimal dew temperature for Ct infection was 20 to 25°C and the favourable condition for disease development was 20/17°C (day/night). A Ct dose higher than $50x10^6$ spores mL^{-1} caused maximum weed suppression. The greatest synergy was observed with Ct plus the herbicide Curtail M or Sencor. Applied alone, the herbicides only suppressed scentless chamomile, whereas their combinations with Ct killed the weed consistently. It was proved that submerged fermentation could be used successfully for mass production of Ct spores. Critical nutrient elements and environmental parameters were established that would facilitate further development of scale-up protocols. A range of formulation additives were evaluated, including nutrient additives, surfactants, humectants, stickers, and oil emulsions, to enhance spray retention, spore germination, and biocontrol performance. Some additives had incremental improvements for Ct infection but these benefits were not strongly demonstrated under field conditions where only Ct in tank mixes with herbicides showed enhanced weed control. DNA sequencing was carried out to reveal molecular differences between chamomile isolates of *C. truncatum* and closely related strains and species which could not be separated reliably based only on fungal morphology. This clarification resolved a potentially contentious issue on crop safety for this biocontrol agent. Overall results of the study suggest that tank mixes of Ct with Curtail M or Sencor have the potential for enhanced control of scentless chamomile in cereal and pulse crops on which feasible post-emergent herbicides are lacking. Continued research on Ct formulation is required to ensure performance of tank mixes under field conditions. Optimization and scale-up of spore production should also be continued to attract commercial interest in adopting this biocontrol technology.

Executive Summary

Scentless chamomile (*Matricaria perforata* Mérat) is a noxious weed, and can be a serious problem in some parts of the province depending on the climate and cropping systems in the area. Woo et al. (1991) reported that 25 plants m^{-2} caused a yield reduction of 55% in spring wheat. Management recommendations, including mechanical and cultural methods, are only partially effective due to the robustness of the weed and changing tillage practices.

Using plant pathogens for weed control is a relatively new approach that has potential to enhance the effectiveness of current management strategies as well as reduce herbicide load in the environment. A project was initiated in 2000 at Agriculture and Agri-Food Canada (AAFC), Saskatoon, to discover and evaluate naturally occurring microorganisms for biocontrol of scentless chamomile. Field surveys were conducted between 2000 and 2002 in Saskatchewan and in the area of weed origin (Europe) for disease causal agents. A total of 706 fungal isolates were assessed, and a group of *Colletotrichum truncatum* (Ct) isolates caused damage to young scentless chamomile without any negative impact on crops. However, application at 200 L/ha resulted in only moderate efficacy on older plants (Figure 1). Although the lesions may expand further and sometime girdle the leaf petiole or stem, the efficacy was considered insufficient because plant kill was generally low.

Figure 1. Disease caused by *C. truncatum* on scentless chamomile

Enhanced weed control can occur when microbial agents are applied in combination with chemical herbicides (Christy et al., 1993). Interactions between two weed control components may vary, but synergism occurs when two or more agents work cooperatively, resulting in greater weed control than each component applied alone. Earlier studies revealed that a number of herbicides interacted positively with the pathogen in question when applied sequentially.

To determine the potential of this biocontrol agent, several questions need to be answered. First of all, can this fungus perform under Saskatchewan conditions? Can its efficacy and consistency be enhanced to meet the requirement of weed control? Can it be mass produced economically? Would it be deemed safe to crops by regulators? This project was aimed to provide research information that addresses these questions. A total of eight studies were conducted and the key findings were highlighted as the following:

Study I. This study looked at the effect of dew temperature, post-inoculation condition and pathogen inoculum dose on infection and disease progress on scentless chamomile, with the objective of determining the optimal infection conditions for the pathogen. Experiments were conducted in growth chamber and greenhouse, and results indicated that the ideal dew

temperature for infection by Ct was between 20 and 25°C. The 20/17°C post-inoculation temperature (day/night) regime was most favourable to disease development, followed closely by the 18/10 °C regime. In Saskatchewan, dew usually occurs when nights are cool, therefore optimal conditions for infection by Ct are infrequent. A formulation that stimulates spore germination and helps sporelings survive adverse conditions would likely be required for field applications of the biocontrol agent. A fungal dose of at least $50x10^6$ spores mL^{-1} was essential for maximum fresh-weight reduction on scentless chamomile. This rate is similar to other *Colletotrichum* bioherbicides reported.

Studies II, III, and IV. These studies were conducted to evaluate interactions between Ct and herbicides for improved control of scentless chamomile by: 1) examining the compatibility of Ct with common herbicides for potential tank mix applications; 2) exploring potential synergy of the fungus with compatible herbicides to enhance weed control; 3) fine tuning herbicide and fungus doses/strain for maximized efficacy and economics.

Most commercial herbicides or surfactants inhibited fungal germination to some extent but this effect was transient over time. Clodinafop, MCPA ester and 2,4-D ester at full label rates were benign to Ct spores while sethoxydim, bentazon, bromoxynil, and glyphosate were most inhibitory. When exposed to herbicides at 0.1x rate, spore germination generally was not affected.

Several herbicides were selected for tank-mix applications based on their compatibility with Ct, modes of action, and efficacy of weed control. More products from Group-1and Group-4 herbicides were examined after initial assessment to determine whether the synergy was a group-related trait and identify the most synergistic herbicide from the group. The nature of Ct-herbicide interaction depends on product efficacy with less efficacious herbicides (Group 1) exhibiting greater 'synergy' with Ct (greater weed control than either the herbicide or Ct alone). Similarly, reduced herbicide rates more frequently showed the synergy with fungus, although the level of weed control was not acceptable by industry standards. In repeated trials, more significant enhancement of weed control occurred with Ct plus the herbicide Curtail M or Sencor, whereas tank mixes with Pursuit, Roundup, Horizon, or 2,4-D were less effective.

The impact by herbicides or Ct alone was greater on younger plants of scentless chamomile, but synergy was identified more readily on older plants where Ct or herbicides were noticeably less efficacious. This may provide choices for tank mix options. For example, scentless chamomile at the 8-leaf stage may be controlled by Curtail M plus Ct or Sencor alone, but older weeds need a tank mix of Sencor and Ct. Additional tank mixtures such as a three-way mix of Curtail M, Pursuit and Ct may be used to control a broad range of weeds including scentless chamomile, in cereal crops. The combination of Sencor and Ct allowed control of scentless chamomile at more advanced growth stages at lower Ct doses.

Study V. Production of Ct spores using submerged fermentation was studied by investigating and optimizing nutritional, physical, and environmental factors. It was revealed that fungus can successfully sporulate in a liquid medium with defined salts plus carbon (C) and nitrogen (N) sources. The process was optimized with screening for most effective nutrient supplies and manipulation of various environmental parameters. Glucose and casamino acid were identified

as the most suitable C and N sources for maximum spore yield and potency. Temperature around 20°C favoured the biomass increase of the fungus but a cooler temperature between 12°C and 16°C was more conducive for sporulation. While aeration (oxygen supply) is important to fungal sporulation, its benefit depends on the available carbon sources. The production may be optimized further by understanding the interaction of these two factors. This study was an additional achievement over our original goals. Although the production process still needs refinement, it is clear that this fungus is capable of producing spores in submerged fermentation. Development of practical scale-up protocols will require further investment in research.

Study VI. Various formulation additives, such as exogenous nutrients, surfactants, stickers, emulsions, and humectants were evaluated to stimulate germination and infection, enhance spray retention, protect germlings, and improve performance. Several adjuvants including Duragel, LI700, Merge, Intac, Score, Glycerol, and Turbocharge enhanced retention slightly, but only Score assisted the fungus most consistently in weed control. Although nutrient additives generally increased spore germination *in vitro*, only molasses showed potential to improve biocontrol performance under controlled conditions. A water-oil-water formulation was used to promote germination under sub-optimal dew conditions and stable emulsions have been created through extensive screening of different vegetable oils and surfactants. These formulation developments, together with synergistic herbicides, formed the basis for field delivery of foliar-applied biocontrol agents.

Study VII. Molecular characterization of the biocontrol agent was conducted using DNA sequencing. There was a concern about using *C. truncatum* for the biocontrol of scentless chamomile in western Canada because the same fungal species also infects lentil, pea, and soybean, causing anthracnose. Morphological differences were unreliable to differentiate isolates of *C. truncatum* from different hosts. Fragments of ribosomal DNA extracted from 70 isolates of *Colletotrichum* spp. collected worldwide were sequenced. Of these isolates, forty two were *C. truncatum* either from scentless chamomile, lentil, pea, or soybean. Results clearly differentiated *C. truncatum* isolates from varying hosts based on nucleotide homology, and those from scentless chamomile were effectively separated from others with significant nucleotide divergences. No variability was found with chamomile isolates from western Canada and Europe, implying that the two populations might have shared a common ancestor. The indigenous pathogen might have come with scentless chamomile to North America when the weed was introduced from Europe at the beginning of last century. This information helps vindicate this biocontrol agent as a potential risk to pulse crops.

Study VIII. Selected biocontrol formulations were tested in tank mixtures with the herbicide Curtail M or Sencor under field conditions in 2004 and 2005 in either pure stands of scentless chamomile or plots seeded with lentil crops. The biocontrol agent caused only light infection on scentless chamomile due to unusually cool temperatures (often below 10°C at night), therefore resulting in no substantial weed suppression. Tank mixing Ct with the herbicide Curtail M or Sencor was generally more efficacious because the fungus was able to take advantage of weed predisposition by the herbicides and disease often progressed into more severe forms of damage. Substantial yield-reduction potential by scentless chamomile was shown in lentil plots. Similar to that found with spring wheat, 25 plants of scentless chamomile per m^2 reduced biomass of

lentil by more than 50% and timely control of the weed is likely of economic benefit. More effective formulations are required for this biocontrol agent to perform consistently under field conditions. The current formulation needs further improvements and the research is being continued with a separate project.

Overall, all the project goals have been met and several additional achievements were made, including investigations on mass production and molecular characterization of the biocontrol agent. Ten public presentations were made during the project to disseminate information. Pertaining to the project, a total of 6 abstracts and 2 papers have been published in conference proceedings. One scientific manuscript has been published, and two more are in press. Three M.Sc. students have been supported by the project funding, with one graduated and the other two in the process of completion. The project stayed within the budget despite two additional activities. The financial statement attached in Appendix 2 provides a summary of expenditures.

Technical Report

Three objectives were proposed for the project to: 1) explore potential synergy between the biocontrol agent *Colletotrichum truncatum* and chemical herbicides to develop feasible tank-mix options for enhanced weed control; 2) develop a delivery system with proper biocontrol formulations that facilitate the application and performance of the fungal agent; 3) validate and demonstrate weed control efficacy under variable field conditions. With the approval by ADF, two additional activities were added in 2004 and 2005 to explore submerged fermentation for potential mass production and clarify taxonomy of the biocontrol agent based on molecular traits to differentiate it from closely related crop pathogens. A total of eight studies were conducted and they are reported individually in the following.

General materials applicable to all studies

Fungal inoculum: An indigenous isolate of *C. truncatum* (Ct) obtained from diseased stem of scentless chamomile was used throughout. For initial testing under controlled conditions, fungal conidia (spores) were produced on V8-juice agar at 13°C for 3 weeks. For field trials, spores were produced in liquid medium containing defined salts plus glucose and casamino acids as carbon and nitrogen sources.

Plants: Seeds of scentless chamomile were planted in a soil-less growth medium in a seeding flat, and seedlings at the 2-leaf stage were transplanted individually and kept in a greenhouse until use. Each pot with one plant formed an experimental unit.

Spray application: Treatments were applied using a cabinet sprayer fitted with a TeeJet XR-8002 nozzle calibrated to deliver 200 L ha^{-1} at 275 kPa. Treated plants were placed in a dew chamber with 100% relative humidity at specified temperatures for 24 hours, then transferred to varying post-inoculation conditions depending on the experiment.

Assessment of disease and weed control: Infection of scentless chamomile was determined using disease severity based on a 0-6 scale representing 0 to 100% disease on the foliage two

weeks after treatment. Plant fresh weight was taken as an indicator of weed control. Disease severity and fresh weight had similar trends, therefore only the later was presented in results.

Experimental design and data analysis: All experiments in controlled environment used a completely randomized design with 3-4 replicates while those under field conditions used a completely randomized block design with 4 replicates. Each experiment was conducted twice unless otherwise stated. Data were subjected to analysis of variance (ANOVA) using SAS, and the normality was examined with PROC UNIVARIATE. Data from repeated trials were pooled when variances were homogeneous, otherwise analyzed separately. Percentage data were transformed with arcsine square root to improve the distribution but non-transformed means were presented. In all cases means were separated with Fisher's protected LSD ($P = 0.05$).

Study I. Effect of dew temperature, post-inoculation condition and pathogen dose on infection of scentless chamomile by *Colletotrichum truncatum*

The objective of this study was to evaluate the effect of environmental factors on infection of scentless chamomile by *C. truncatum*. This information will identify the optimal conditions for biocontrol activities.

Materials and Methods

Experiment 1

Two trials were conducted separately to examine the effect of low and high dew temperatures on infection. The temperature range was similar to those reported for other *Colletotrichum* bioherbicides. In addition, typical spring temperatures encountered on the Canadian prairie were also taken into consideration.

Effect of low dew temperature and varying post-inoculation conditions: Two dew temperatures, 15 and 20°C, and three post-inoculation conditions were used: the greenhouse, a growth chamber set at 20/17°C (day and night) and a similar growth chamber at 18/10°C. The day length in both growth chambers was 14 hours. The 20/17°C cycle was used to mimic the temperature regime in the greenhouse. The 18/10°C cycle was chosen to simulate the average early spring temperatures in Saskatchewan. Three spore doses were applied, 0, $2x10^6$ and $10x10^6$ spores mL^{-1}, with the no spore treatment as the control.

Effect of high dew temperature and varying post-inoculation conditions: Two dew temperatures, 25 and 30°C, were tested in combination with three post-inoculation conditions: the greenhouse and growth chambers at 30/17°C and 20/17°C. The 30/17°C cycle was selected to favour disease progress under higher temperatures. Only two spore doses, 0 and $10x10^6$ spores mL^{-1}, were applied in this experiment due to only slight disease severity at the lower pathogen dose.

Experiment 2

Effect of Ct dose under varying post-inoculation condition: After determination of the optimum temperatures, this experiment was conducted to examine the effect of spore dose using 25°C dew temperature and 20/17°C post-inoculation temperature in the greenhouse and growth

chamber settings as described earlier. Six spore doses were applied, i.e., 0, $1x10^6$, $25x10^6$, 50 $x10^6$, $75x10^6$, $100x10^6$ spores mL^{-1}.

Experimental design

Experiments including different dew temperatures and post-inoculation conditions used a split-split plot design. The main plots, the post-inoculation condition, were replicated twice. The sub plots and sub-sub plots consisted of dew temperatures and Ct doses, respectively. Each sub-sub plot treatment was replicated four times. The experiment involving six spore doses was a split-plot design with post inoculation condition as main-plot and Ct dose as sub-plot factors. The main plots were replicated twice and the sub plots were replicated four times.

Results and Observations

Experiment 1

Effect of dew temperature and varying post-inoculation conditions: Under low dew temperatures, Ct treatments reduced the fresh weight by 11% and 14% under 15°C and 20°C dew temperatures, respectively, but the efficacy between the two Ct doses was similar (data not shown). Under higher dew temperatures, Ct treatment was more effective at 25°C than at 30°C, reducing the fresh weight of chamomile by about 30% when compared to untreated controls.

Experiment 2

Effect of Ct dose under varying post-inoculation condition: There was no efficacy difference between greenhouse and growth chamber conditions (post-inoculation), therefore data from the two conditions were pooled. An increase in Ct dose generally showed higher weed suppression (Figure I-1). For maximum efficacy, a Ct dose at $50x10^6$/ml was required.

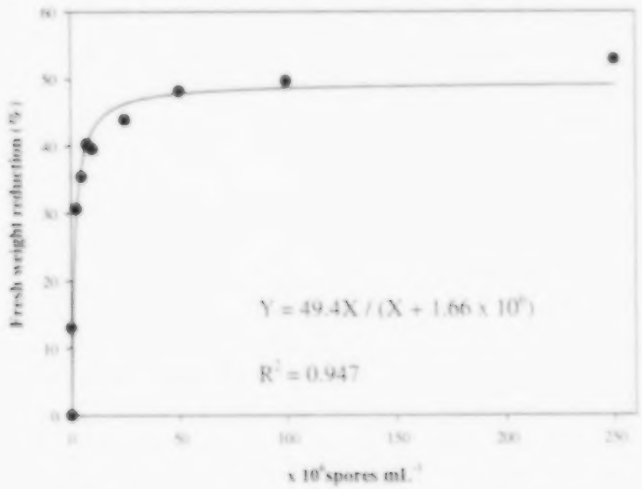

Figure I-1. Regression of fresh weight reduction on scentless chamomile by *Colletotrichum truncatum* applied in varying concentrations at the carrier volume of 200 L ha^{-1}. Data was from 6 trials with varied spore concentrations.

Conclusions

The ideal dew temperature for Ct infection is between 20 and 25 °C, and the 20/17°C post-inoculation temperature (day/night) was most favourable to disease development. A pathogen dose of $5x10^7$ spores mL^{-1} will be required for maximum impact on scentless chamomile. In Saskatchewan, typical conditions in early spring likely are not conducive to infection, and formulation will be required to promote infection under field conditions.

Study II. Interaction between *Colletotrichum truncatum* and herbicides for synergy in control of scentless chamomile.

Objectives: 1) Assess the compatibility of Ct with commonly used herbicides *in vitro* for tank mix applications of the two components; 2) evaluate compatible herbicides in different tank mixtures with Ct for improved control of scentless chamomile; 3) Determine weed-control synergy between Ct with herbicides from selected families.

Materials and Methods

Experiment 1. Herbicide effect on spore germination

Herbicide selection: Sixteen products from seven herbicide groups were chosen for this test. Score (0.8% v/v), Merge (1 L ha^{-1}) and Agral 90 (0.2% v/v) were also included due to their common uses as spray adjuvants. Whenever possible, each herbicide or adjuvant was tested separately.

Effect of herbicides/adjuvants on Ct spore germination: A mixture of spores with a herbicide or adjuvant was spread on potato dextrose agar in Petri plats. Two rates of each product, 1x and 0.1x, were used according to label instructions. Water was included as a control. At the end of 10-h and 24-h incubation at 21°C, each replicate was stained with 0.01% lactophenol cotton blue and percent germination was determined by counting 100 spores. Differences of means were determined with one-tailed $LSD_{0.05}$ as only the germination lower than that of the control was of interest.

Experiment 2. Interaction of Ct with herbicides on scentless chamomile

Ct spores were applied as tank mixtures with selected herbicides to scentless chamomile at the 8-10 leaf stage. The pathogen dose at $7x10^6$ spores mL^{-1} was used. Herbicides and surfactants (when applicable) were prepared at concentrations based on recommended field-application rates and volumes. Each herbicide was tested at 1x and 0.1x rates with or without Ct. The nature of interaction was determined using a method introduced by Colby (1967).

Colby's method: This is one of the common methods used to determine if interactions of herbicides are cooperative. The calculation is based on the efficacy of single components in comparison to that by the mixture. In this study, observed weed control by a tank mix was compared with expected responses using protected $LSD_{0.05}$, as synergistic (greater), antagonistic (lower), or additive (no significant difference).

Initial assessment of herbicide/Ct tank-mixes: Seven herbicides, most of them were benign to Ct spores, were tested with the fungus for control of scentless chamomile. These products represented a range of modes of action. Glufosinate and bentazon were dropped after initial trials due to high efficacy under the greenhouse condition, masking any benefits from Ct.

Synergy between Ct and selected herbicide groups: Based on initial results, two herbicide groups (1 and 4) were explored further to identify most effective products for tank mixes with Ct. Five herbicide from each of the Group 1 and 4 categories were selected to represent a cross section of products used in the prairies.

Results and Observations

Experiment 1

Herbicide effect on spore germination: At 10 h, about 59% of Ct spores germinated in the control, whereas the germination varied with herbicides or adjuvants. Glufosinate, clodinafop, MCPA and 2,4-D ester were least inhibitory, while all the adjuvants as well as the herbicide bentazon, sethoxydim, glyphosate and bromoxynil at 1x were highly inhibitory. By 24 h, the germination was no longer affected by most chemicals.

Experiment 2

Effect of Ct-herbicide tank mixtures on scentless chamomile: MCPA alone at 1x showed suppression on scentless chamomile but the efficacy was increased significantly by adding Ct in tank mix. Metribuzin, imazethapyr and glyphosate all suppressed scentless chamomile, but only metribuzin plus Ct caused greater weed control. None of the Group-1 herbicides plus Ct resulted in satisfactory weed control. Clopyralid was the most synergistic Group-4 product, killing scentless chamomile at the 8-leaf stage when tank mixed with Ct (Table II-1).

Table II-1. Effect of Ct plus Group-4 herbicides on fresh weight (g) of scentless chamomile.

Herbicide	Ct absent	Ct present	Expected value [a]	Interaction [a]
Control	7.1 a [b]	4.5 b		
MCPA ester (1x)	4.0 b	2.2 c	2.5	Additive
MCPA ester (0.1x)	6.4 a	3.4 b	4.0	Synergistic
2,4-D ester (1x)	2.9 b	1.9 c	1.8	Additive
2,4-D ester (0.1x)	5.9 a	3.0 b	3.7	Synergistic
clopyralid (1x)	2.6 b	1.2 c	1.6	Synergistic
clopyralid (0.1x)	4.3 a	2.3 b	2.7	Synergistic
2,4-D amine (1x)	3.0 bc	2.4 c	1.9	Antagonistic
2,4-D amine (0.1x)	5.3 a	3.6 b	3.3	Additive
dicamba (1x)	3.5 b	3.0 b	2.2	Antagonistic
dicamba (0.1x)	6.6 a	3.7 b	4.2	Synergistic

[a] Based on Colby's formula and definition.

[b] Mean separations applicable within herbicides ($P<0.05$). $LSD_{0.05}$ = 1.0 g.

Conclusions
Most significant enhancement of weed control was observed with tank mixtures of Ct with the herbicide Clopyralid (Group-4) or Metribuzin (Group-5).

Study III. Synergy of Ct-herbicide mixtures on scentless chamomile at different growth stages

The objectives were to: 1) evaluate effect of selected Ct-herbicide combinations on scentless chamomile at varying growth stages; 2) determine the benefit of combining herbicides of different modes of action in tank mixtures with Ct; and 3) assess the effect of Ct doses in tank mixtures with herbicides.

Materials and Methods

Experiment 1. Varying Ct-herbicide combinations
Plants of scentless chamomile were treated at 8- and 11-leaf stages. Clopyralid plus MCPA (Curtail M), Curtail M plus clodinafop (Horizon), and metribuzin (Sencor) were tank mixed with Ct at $7x10^6$ spores mL^{-1}. In earlier experiments, MCPA alone was insufficient against scentless chamomile even with Ct, but combining with clopyralid (Curtain M) is a common mix for controlling broadleaf weeds in cereal crops. The mixture of Curtail M and Horizon targets a wide range of broadleaf and grass weeds in cereals. Previously in the Study II, Sencor in a tank mix with Ct effectively controlled scentless chamomile.

Experiment 2. Effect of Ct doses
Four herbicides, i.e., Horizon, 2,4-D, Curtail M and Sencor, were tested at 1x label rates with 4 Ct doses on scentless chamomile at 8- and 11-leaf stages. Ct spores were suspended in herbicides at 0, $2x10^6$, $7x10^6$ and $20x10^6$ mL^{-1}. The $7x10^6$ mL^{-1} concentration was a standard Ct dose used previously. The minimum dose of $2x10^6$ spores mL^{-1} was required to cause a measurable level of damage by Ct alone. It was hypothesized that herbicides would allow to reduce Ct doses without compromising weed control.

Experiment 3. Synergy of Sencor at reduced rates
Ct was most efficacious when tank mixed with the herbicide Sencor at 1x rate. This trial was to explore the synergy at reduced herbicide rates. Scentless chamomile at 8-10 and 11-13 leaf stages were used. The label rate for Sencor (1x) was 0.208 kg a.i./ha, and the reduced rate was 0.5x the label rate. The experiment was factorial (herbicide rate x growth stage).

Experiment 4. Evaluate the synergy of Pea Pack (metribuzin + MCPA) with Ct.
Pea Pack is a convenient pre-packaged tank mix of Metribuzin and MCPA used commonly in pulse crops. Individually, both products were synergistic with Ct on scentless chamomile. The objective was to assess if the pack mix would be more synergistic.

Results and Observations

Experiment 1. Synergy with Ct-herbicide combinations

Combining Ct with the herbicides improved weed control, especially on older plants (the 11-leaf stage). On younger plants (8-leaf), however, herbicides were quite effective and Ct did not consistently provide a synergistic benefit.

Curtail M plus Ct significantly increased weed control at the 8-leaf stage, representing an 82 % fresh-weight reduction as compared to the non-treated controls and a 56 % improvement over the herbicide alone. A similar trend was seen at the 11-leaf stage with noticeably lower efficacy. Adding clodinafop to Curtain M and Ct tank mix did not further increase weed control. Sencor alone was sufficiently effective on younger plants. On older plants, however, the herbicide alone reduced the fresh weight by 60% but no plants were killed. Tank mixing Ct with Sencor increased efficacy by 35 % when compared to the herbicide alone (Table III-1), and killed scentless chamomile plants consistently.

Table III-1. Fresh weight (g) of scentless chamomile as affected by Sencor plus Ct treatments.

Chamomile	Herbicide	Ct absent	Ct present
8-leaf	Control	7.2 a [a]	5.6 b
	Metribuzin	0.3 c	0.3 c
11-leaf	Control	12.3 a	10.0 b
	Metribuzin	4.3 c	2.9 d

[a] Mean separations applicable within the same leaf stage, and treatments with the same letters are not significantly different ($P<0.05$). $LSD_{0.05} = 1.3$ g.

Experiment 2. Effect of Ct dose

When tank mixed with Curtail M, higher doses of Ct had greater efficacy and killed most of the plants at the 8-leaf stage but not older plants. Sencor alone was effective on younger plants (Table III-2). At the 11-leaf stage, herbicide plus Ct at higher than $2x10^6$ spores mL^{-1} rates were more efficacious than the herbicide alone, with more consistent plant kill.

Experiment 3. Sencor at reduced rates.

Only treatments with 1x Sencor plus Ct had high efficacy on scentless chamomile at the 11-leaf stage (Table III-3), killing plants consistently. On a few occasions, Sencor at 0.5x plus Ct killed younger plants. The herbicide at 1x rate is likely required for tank mixing with Ct in field uses. In comparison with Sencor alone, no benefit in weed control was observed with the Pea Pack (Sencor plus MCPA). There was no enhancement of efficacy or synergy with Ct (data not shown).

Table III-2. Effect of Sencor plus Ct at varying doses on fresh weight (g) of scentless chamomile at two growth stages.

Chamomile	Herbicide	Ct dose (spores mL^{-1})			
		0	$2x10^6$	$7x10^6$	$20x10^6$
8-Leaf	Control	8.0 a[b]	7.0 ab	6.0 bc	5.4 c
	Sencor	0.5 d	0.4 d	0.4 d	0.4 d
	Expected value [a]		0.6	0.5	0.4
	Interaction [a]		Additive	Additive	Additive
11-Leaf	Control	11.3 a	10.4 a	10.1 ab	8.9 b
	Sencor	5.3 c	4.5 cd	2.6 e	3.4 de
	Expected value		4.9	4.8	4.2
	Interaction		Additive	Synergistic	Synergistic

[a] Based on Colby's Method

[b] Mean separations applicable within the same leaf stage, and treatments with the same letters are not significantly different ($P<0.05$). ($LSD_{0.05}$ = 1.5 g

Table III-3. Effect of Sencor at varying rates plus Ct on fresh weight (g) of scentless chamomile at different growth stages.

Plant stage	Ct spore dose ($x10^6$ /ml)	Sencor rate		
		0x	0.5x	1x
8-leaf	0	7.4 cde [b]	4.5 fg	0.8 i
	7	6.2 de	2.7 h	0.5 i
11-leaf	0	10.4 a	9.1 ab	3.9 gh
	7	7.7 bcd	6.6 de	2.9 h

[b] Means with the same letters are not significantly different ($LSD_{0.05}$).

Comments and Conclusions

Due to variations in weed populations, it is import to understand the performance of biocontrol agents at different growth stages of weeds. Combining Ct with different herbicides resulted in variable response from scentless chamomile, and tank-mix applications should take the weed growth stage into consideration. For instance, Curtail M plus Ct may be sufficient for control of scentless chamomile at the 8-leaf stage, but this mix is less effective on plants at the 11-leaf stage. A mix of Sencor with Ct may still be able to control the older weed satisfactory.

In general, as Ct dose increased, so did the weed suppression but the efficacy by the pathogen alone was insufficient for acceptable weed control. When combined with herbicides, higher Ct doses may be required for less efficacious herbicides, whereas less Ct spores are needed if in tank mix with a highly efficacious product such as Sencor.

Weed control or suppression by both herbicides and the biocontrol agent Ct were greater on younger plants of scentless chamomile, whereas synergy was identified more often on older plants due to reduced Ct and herbicide efficacy. While Curtail M may be used in a tank mix with Ct on younger scentless chamomile, Sencor plus the fungus is more efficacious on older plants (Appendix 1, Photo 1&2). Curtail M is more effective when mixed with Ct at high doses, whereas Sencor can be applied with Ct at moderate doses.

Study IV. Control of scentless chamomile by herbicides plus different Ct isolates

The objective of this study was to assess interactions of synergistic herbicides with different Ct isolates obtained in Saskatchewan.

Materials and Methods

Isolates

Six Ct isolates, obtained from different regions in the province during field surveys between 1999 and 2001, were used. Most isolates showed similar virulence on scentless chamomile but the isolate 00-248A1 and 99-21A1caused lighter disease in previous trials. The isolate 00-248A1 was also able to infect flax and lentil.

Treatments

Horizon, 2,4-D, Curtail M, or Sencor were applied in tank mixes with spores of the selected isolates to scentless chamomile at 8- and 11-leaf stages. The herbicides' was applied at 1x label rate at 200 L/ha, and approximately $7x10^6$ spores mL^{-1} concentration was targeted for different Ct isolates.

Results and Conclusions

Isolate 00-3B1

As in other trials, the isolate 00-3B1 reduced the fresh weight of scentless chamomile at both leaf stages (Table IV-1). At the 8-leaf stage, most herbicides showed little synergy with the isolate, except the Horizon. On older plants, herbicide plus Ct increased efficacy as compared to the herbicide or Ct alone. Sencor plus Ct was the most effective treatment, with 50 % more

fresh weight reduction and consistent weed kill when compared to the herbicide alone. The isolates 00-193C, 01-40 and 01-60 showed a similar trend with the herbicides (data not shown).

Table IV-1. Effect of the Ct isolate 00-3B1 in tank mixes with herbicides on fresh weight (g) of scentless chamomile at different growth stages.

Growth stage	Herbicide	Ct absent	Ct present	Expected value [a]	Interaction [a]
8-leaf	None	6.6	4.8 ** [b]		
	Horizon	6.8	4.5 **	4.9	Synergistic
	2,4-D	2.9	2.1	2.1	Additive
	Curtail M	1.7	1.1	1.2	Additive
	Sencor	0.8	0.7	0.6	Additive
11-leaf	None	10.7	9.1 **		
	Horizon	10.7	7.8 **	9.0	Synergistic
	2,4-D	6.2	5.7	5.2	Additive
	Curtail M	5.2	4.0 **	4.4	Additive
	Sencor	3.4	1.7 **	2.9	Synergistic

[a] Based on Colby's Method

[b] t-test between Ct absent and present for a given herbicide. * and ** indicate a significant difference at $P < 0.05$ and < 0.01. Otherwise, there is no significant difference ($P > 0.05$).

Isolate 99-21A1 and 00-248A1

These two isolates appeared less virulent than others in this study. Although tank mixing with the herbicides increased fresh-weight reduction, the efficacy was lower than the tank mixtures using other isolates and no plants were killed at the 11-leaf stage. The isolate 00–248A1 was least virulent among all isolates tested with generally insignificant effect on fresh weight of scentless chamomile. In most cases, mixes of herbicide with the isolate 00–248A1 did not improve weed control significantly.

Conclusions

Evidence suggests variation in virulence to scentless chamomile exists in *Colletotrichum* populations, and there is a need and opportunity to evaluate a range of isolates within a target species for individuals with most fitted traits. Several isolates showed similar efficacy to that of 00-3B1, but none of them was significantly superior. In combination with Sencor, the isolate 00-3B1, 01-40 and 00-193C showed a similar level of weed control, resulting in greater that 80% of fresh-weight reduction (Figure IV-1), and killed the majority of older chamomile plants.

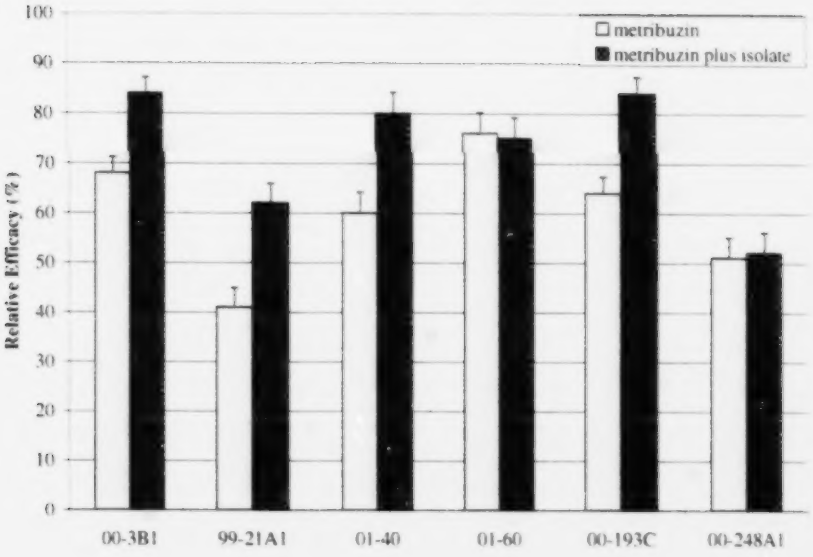

Figure IV-1. Percent fresh-weight reduction (relative efficacy) by Sencor (metribuzin) alone or Sencor plus different Ct isolates on scentless chamomile at the 11-leaf stage.

Study V. Production of Ct spores using submerged culture fermentation

The objective: investigate the possibility of using liquid fermentation for mass production of *C. truncatum* spores and identify optimal fermentation parameters for yield and potency.

Materials and Methods

Shake-flask studies

Fungal inoculum: The isolate 00-3B1was used throughout this study. Stock cultures were transferred on V8-juce agar and sporulating colonies flooded with sterile water to make spore suspensions (seed inoculum) for inoculation of liquid media in flasks.

Treatment assessment: Spore yield was measured after 2 to 3 weeks. The potency of the spores was determined by inoculating plants of scentless chamomile at the 8-leaf stage using an airbrush sprayer then rated a week later for disease severity and fresh-weight reduction using the criteria described earlier.

Effect of nutrient amendments: A defined basal salts medium (DBSM, Jackson et al., 1996) was amended with 9 different carbon (C) sources and 8 nitrogen sources, separately, with a C:N ratio of 23:1. Cultures were incubated at constant 16°C, and effects of varying C concentration and C:N ratio were also assessed.

Effect of physical and environmental factors: DBSM amended with glucose (10 g/L) and casamino acids at 20:1 C:N ratio was used as a standard liquid medium. Several experiments were conducted to determine the optimal initial inoculum concentration, pH, aeration, light, and temperature regimes (16 to 28°C) during fermentation.

Preliminary scale-up trials and refinements of protocols

Production runs using bench-top fermenters: The same DBSM amended with glucose and casamino acids and C and N sources was utilized for these trials. The working capacity of bench-top fermenters (Appendix 1, Photo 3) ranged from 10L to 15L, and the medium was inoculated with 200 ml of seed inoculum (1.5×10^7 spores/ml) produced in shake flasks to reach an initial concentration of 10^3 spores/ml. The following temperature regime was used: 22°C for the 1st week and 12°C for the 2nd week. Two or three dissolved oxygen (dO) levels were maintained during production with a dO cascade of varying agitation speeds. Glucose concentration in the medium (indication of carbon exhaustion) was monitored at 24-h intervals and biomass taken at 48-h intervals.

Interaction of carbon concentration and aeration: This additional experiment used shake-flask cultures to test a hypothesis that high dO will not enhance Ct sporulation when carbon supplies are inadequate. Cultures were incubated on rotary shakers at 16°C. Varying levels of aeration (simulating dO) were created by using standard Erlenmeyer flasks at slow (75 rpm) vs. Baffled flasks at fast (150 rpm) shaker speeds. It was estimated that Baffled flasks provide 15% greater aeration than standard flasks.

Results and Conclusions

Shake-flask studies

Effect of nutrient amendments: Spore yield was higher in DBSM supplemented with glucose and casamino acids as C and N sources, when compared to V8-juice broth. Cooler temperatures (12 to 16°C) were more favourable to trigger sporulation as opposed to 28°C. Spores produced in DBSM were as virulent as those produced on V8-juice agar.

Sporulation did not occur when molasses was used as a C source, and was poor with fructose or glycerol. Trehalose, sucrose, lactose, and maltose supported higher spore yields but the highest sporulation occurred in the medium with glucose as the C source. Glucose has been reported as a superior C source for sporulation of other fungal biocontrol agents in submerged cultures (Jackson et al. 1990; Zhang et al. 2001). DBSM with casein hydrolysate or casamino acid as the N source produced higher numbers of spores, while sporulation was low with other N sources. Casamino acid was selected for further studies due to its clearly defined nutrient composition.

There was no biomass increase or sporulation in DBSM containing 0 g of glucose. Cultures with higher C concentrations (25-40g glucose/L) tended to produce greater numbers of spores. There was also a slight tendency that lower C concentration favoured virulence of spores for infection of scentless chamomile. The C:N ratio within the range selected did not affect spore yield or virulence substantially, with a slight tendency of higher spore yields around 20:1.

Effect of physical and environmental factors: Higher spore yields were seen with lower initial spore concentrations, especially between 10^2 and 10^3 spores/ml. No consistent pattern of yield differences can be determined between pH ranging from 4.5 and 6.5, but the spore yield at pH 7.5 was twice as much as that at 4.5. There was high variability with the data. It was not known whether pH levels higher than 7.5 could be more favourable. Medium pH did not affect the virulence of spores produced

This fungus sporulated equally well under a range of dark/light settings. This insensitivity to lighting is a positive trait for large-scale production where strict control can be more difficult. Higher yields occurred with the variable temperature regime in which the temperature was lowered from 20°C to 12°C in the second week of fermentation. Constant temperatures at 12°C and 20°C were less favourable. It is believed that this dip in temperature might have triggered the sporulation process, helping shorten the fermentation cycle. The fermentation temperature had no significant effect on Ct virulence. Cultures in Baffled flasks which had higher aeration or dissolved oxygen (dO) yielded 3 times more spores than those in regular Erlenmeyer flasks. For further manipulation of dO, a wider range of settings can be achieved with computerized fermentors.

Scale-up trials and fermentation parameter refinements

Production using bench-top fermenters: Sporulation was noticeably lower with fermentor runs and there was an increased amount of microsclerotia in the culture, especially under high dO conditions. There were no substantial differences in fungal biomass or spore yield relative to dO levels tested.

Additional notes from scale-up trials: 1) lowering fermentation temperature failed to prompt profuse sporulation as observed in shake-flask cultures and additional triggers may need to be investigated; 2) overproduction of microsclerotia presented a problem for spore harvest using conventional centrifugation technologies; 3) the capability of fermentors to provide high dO resulted in rapid depletion of carbon sources in the medium, which limited growth of fungal biomass and sporulation, glucose was completely consumed under all dO conditions within 5 days. Biomass and spore production levelled off shortly after sugar exhaustion. With more dO, higher C concentrations may help sustain the increase of Ct biomass and greater spore production. A shake-culture experiment was carried out later to examine this hypothesis through interactions of aeration and C concentration. Spores produced with fermentors were equally efficacious in causing disease on scentless chamomile.

Two trials using shake-flask cultures largely confirmed our hypothesis. There was a strong interaction between the C concentration and aeration. Higher aeration in Baffled flasks plus faster shaker speed did not provide benefit to spore production unless >20 g/L glucose was used in the medium (Figure V-1). The greatest number of spores was produced consistently at the higher aeration supplied with 40 g/L glucose. This sugar concentration was 4 times higher than that used in most of the previous trials including scale ups. At the lower aeration, however, increasing glucose concentration did not improve spore yields. The dO level and sugar concentration can be further optimized for scale-up productions.

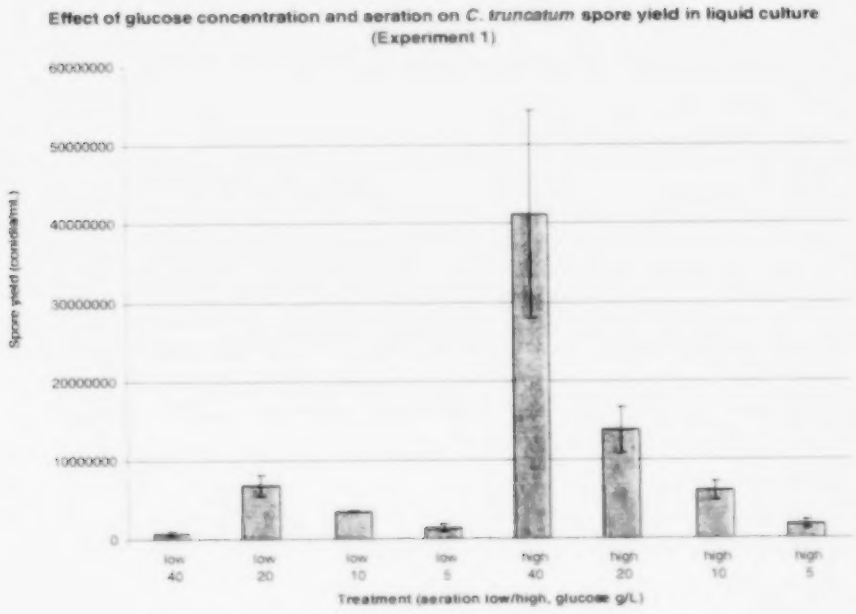

Figure V-2. Ct spore yields affected by aeration and glucose concentration in liquid medium.

Conclusions

This study demonstrated that Ct spores can be successfully produced in submerged culture for biocontrol of scentless chamomile. The DBSM can be further amended with optimal amounts of C and N for maximum yield and potency. So far glucose and casamino acid appeared to be the most favourable C and N sources. Temperature around 20°C favoured Ct biomass growth, but a cooler temperature between 12°C and 16°C was more conducive for sporulation. While aeration is important to the sporulation, its benefit depends on C supplies in the medium and spore yield may be optimized by further investigation of interactions between the two factors.

With limited resources, we have only scratched the surface of Ct liquid fermentation. This is an additional achievement over our original goals but it is important to understand the probability of mass production using a typical industrial system. Although the process can be improved further, it is clear that the fungus is capable of producing a large number of spores in liquid cultures. Development of feasible scale-up protocols requires further investment in research.

Study VI. Develop formulations to enhance Ct performance

Background

Previous studies suggested that extremely high Ct spores doses are needed for substantial suppression of scentless chamomile. The fungus also requires wetness (dew) duration up to 20 hours for infection. These requirements present a challenge for use of this biocontrol agent under field conditions due to the cost and weather limitations on the prairies. The efficiency of spray retention affects the rate and performance of herbicides, and various adjuvants have been used to improve applications as well as weed control efficacy. For microbial agents, the speed of fungal infection is affected by the promptness of spore germination and penetration that are impacted largely by the free moisture, temperature, and essential nutrient elements on plant surfaces. This study examined a range of additives as potential formulation ingredients to enhance spray retention, infection, and biocontrol efficacy.

Material and Methods

Experiment 1. Evaluate adjuvants to improve spray retention on scentless chamomile

Initially, a total of 26 adjuvants representing a diverse range of functional groups such as surfactants, stickers, emulsifiers, and humectants were evaluated at 1.0% and 0.1% concentrations (v/v or w/v). The retention efficiency on scentless chamomile was estimated using a previously developed protocol (Peng et al. 2005). A dye was added to liquids and the amounts recovered from plants were used as the indicator of retention volumes. For each adjuvant concentration, six plants were used for replication. Eleven adjuvants with varying retention effects were further evaluated with Ct on scentless chamomile. Three trials were conducted with a total of 12 replicates for each adjuvant.

Experiment 2. Evaluate nutrients for enhancement of Ct germination and virulence

Ct spores require exogenous nutrients for high percentage of germination. The following defined and complex nutrients were tested for germination promotion:

Table VI-1. Nutritional additives in Ct spore suspensions for germination promotion.

Carbon source	Nitrogen source	Complex	Leaf extract
Fructose	Alanine	Molasses	Scentless chamomile
Glucose	Casamino acid	Potato-dextrose broth (PDB)	Lentil (cv. Eston)
Sucrose	Proline	Sorbitol	Wheat (AC Crystal)
Trehalose	Peptone	Starch	
		Yeast extract	

Three separate trials were conducted. First, all nutrient elements were tested at 1% (w/v or v/v) concentration, except that PDB was used at 2%. Several nutrient additives deemed beneficial to germination were further tested at 2x and 4x higher as well as 0.5x, 0.25x, and 0.13x lower concentrations. In the third trial, molasses, PDB, and chamomile leaf extracts were added to Ct spore suspensions for inoculation of chamomile plants at the 8-leaf stage.

Experiment 3. Water/oil/water (WOW) Formulation

The purpose of this trial was to formulate Ct in an oil-based emulsion that would potentially extend wetness duration and also could be applied using common spray equipment. Several emulsifying agents were assessed for compatibility with Ct and stability of formulations. Ct spores were suspended in water amended with a surfactant, then added to an oil-surfactant mixture to produce an invert emulsion that was later suspended in a surfactant solution to form a WOW formulation. Stability of the formulation was determined according to separation of oil and water and mean diameters of emulsion droplets over a 15-day period. The effectiveness of the formulation was evaluated based on disease severity induced under varying conditions.

Results and Conclusions

Experiment 1. Adjuvant Evaluation

In comparison to controls (no additives), most adjuvants did not substantially increase spray retention. Duragel, LI700, Merge, Intac, Score, Glycerol, and Turbocharge enhanced retention only slightly. However, when tested with Ct spores on scentless chamomile, only Score was consistently beneficial, increasing weed control slightly when compared to Ct suspended in water (Table VI-1). The mechanism of adjuvant was unclear but likely may not be related to retention because Score did not result in the highest retention efficiency among the products tested.

Experiment 2. Effect of nutrients on Ct germination and infection

Without adding nutrients, only 10% of Ct spores germinated. Molasses and chamomile leaf extracts enhanced germination most substantially. Simple C or N additives such as sucrose, trehalose, proline, peptone, or casamino acid, did not increase spore germination. Molasses at 0.25% was enough to increase spore germination and weed suppression by Ct (Table VI-2).

Experiment 3. WOW formulation

Formulation stability: For water-canola invert emulsions, ATSURF595-Tween80, Span80-Tween60, and Span80-Tween85 provided most stability among 19 surfactant mixtures evaluated (Figure VI-1) while Span 85-Tween 20 and Span 85-Tween 40 did not emulsify water and canola oil. The greatest change in the stability was within the first 5 days for all the emulsions but very little to no change was observed after that. Ct spores were most compatible with Span80-Tween60. For the crude soybean oil-water emulsification, Span 80-Tween80 or Span 85/20-Tween 80 were optimum surfactants.

Table VI-2. Effect of Ct amended with adjuvants on scentless chamomile fresh weight (g).

Adjuvant	Ct absent	Ct present
Control (water)	9.3 ab [a]	7.2 ghij
Durajel 1.0%	9.0 abcd	6.6 ijk
Ekol 1.0%	8.8 bcd	6.6 ijk
Glycerol 0.1%	9.6 ab	7.4 fghi
Intac 1.0%	9.1 abc	7.1 hij
LI700 1.0%	9.8 a	7.8 efgh
Merge 0.1%	8.7 bcde	8.1 defg
Prevail C 1.0%	8.9 abcd	8.3 cdef
Score 0.1%	8.7 bcde	5.9 k
Sorbitol 1.0%	9.0 abcd	6.3 jk
Turbocharge 0.1%	8.9 abcd	7.5 fghi
Tween 80 0.1% (standard)	8.7 bcde	6.7 ijk

[a] Means with the same letter (s) are not significantly different ($P < 0.05$). $LSD_{0.05} = 1.0$ g.

Table VI-3. Fresh weight (g) of scentless chamomile as affected by Ct amended with nutrients.

Treatment	Ct Absent	Ct present
Non-treated control	9.7 a [a]	6.3 b [a]
Molasses (4%)	9.4 a	4.4 c
Molasses (1%)	8.9 a	4.5 c
Molasses (0.25%)	9.0 a	5.1 bc
Chamomile extract (4%, w/v)	8.8 a	5.2 bc
Chamomile extract (1%, w/v)	9.2 a	5.3 bc
Chamomile extract (0.25%, w/v)	9.2 a	6.1 b

[a] Means with the same letter (s) are not significantly different ($P < 0.05$). $LSD_{0.05} = 1.3$ g.

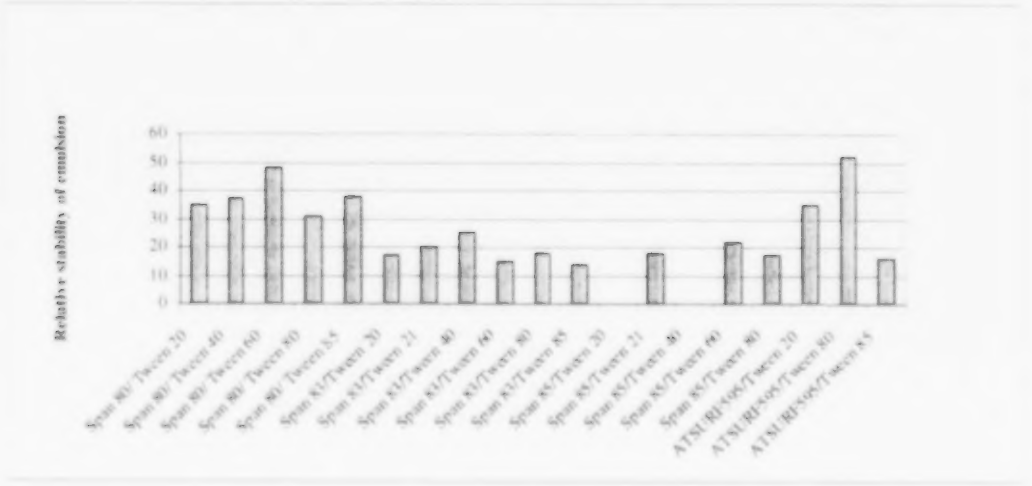

Figure VI-1. Stability of invert emulsions (water-canola oil) made with various surfactants.

Study VII. Molecular characterization of the biocontrol agent

Background

While *Colletotrichum truncatum* showed promise for biocontrol of scentless chamomile, the same fungal species is also known to cause anthracnose diseases on lentil, pea, and soybean. Classical identification of *Colletotrichum* species is based on conidial and appressorial size and shape, as well as host range, but these morphological differences are often insufficient to differentiate *C. truncatum* from closely related species such as *C. capsici* (Syd.) Butl. & Bisby, *C. linicola* Pethybr. & Lafferty, and *C. circinans* (Berk.) Vogl.

More definitive traits are required to differentiate the biocontrol agent from crop pathogens for risk assessment. Molecular techniques, especially ribosomal DNA (rDNA) sequencing, have been proven to be highly useful in separating closely related *Colletotrichum* species (Sherriff et al. 1994) and intraspecific strains (Ford et al. 2004; Sreenivasaprasad et al. 1996).

Materials and Methods

Mycelia of 70 isolates of *Colletotrichum* spp. collected from a range of hosts worldwide were produced in potato dextrose broth for DNA extraction and sequencing. Of the isolates, forty two were from scentless chamomile (11), lentil (18), pea (5), soybean (7) or Caribbean stylo (1) and were identified as *C. truncatum* or truncatum-like species.

Initially, a fragment of rDNA, which included internal transcribed spacer region 1 and 2 (ITS1, ITS2) as well as the 5.8S rDNA, was amplified with PCR using the universal primers ITS4 and ITS5. All PCR experiments for taxon-specific amplification were performed using standard

protocols. Amplicons were processed with QIAGEN purification kits and samples were sequenced for the two DNA strands.

Sequences of 5.8S rDNA-ITS fragments were aligned using the multiple-sequence alignment program CLUSTAL.X®, and the aligned sequences were loaded to the PAUP® program and grouped with Parsimony and Neighbor-Joining trees supported by bootstraps based on 50% majority-rule consensus (1,000 replications). The size of rDNA fragments sequenced ranged from 560 to 600 base pairs (bp). Sequence analyses were based on nucleotides from 50th to 200th bp for ITS1 and 400th to 550th bp for ITS2, respectively.

Results and Observations

Sequencing results clearly separated *C. truncatum* isolates from other *Colletotrichum* species (Figure VII-1). Within the species *C. truncatum*, chamomile isolates were more closely related to lentil isolates than to soybean isolates based on nucleotide similarity, but there were enough divergence (>4%) that divided chamomile and lentil isolates into separate clusters. Isolates within either the chamomile, lentil or soybean cluster were almost identical (>99% homology). Pea isolates were substantially different from *C. truncatum* and, were divided into two separate clusters with the species *C. circinans* and *C. coccodes*, respectively. No substantial variation was observed with chamomile isolates from Saskatchewan and Europe, implying that the two populations might have shared a common ancestor.

Microscopic observations revealed that conidia of *C. truncatum* isolates from chamomile and lentil were slightly falcate but spores of the chamomile isolate appeared a little more truncated (Figure VII-1 a). Conidia of the soybean isolate were typically falcate, whereas those of alfalfa isolates were ovoid. Appressoria of various isolates were largely globose (Figure VII-1 b).

Conidia of the soybean isolate were approximate 50% longer but 20% narrower than those of other isolates examined (Table VII-1). Additionally, its appressoria were about 40% larger than those of other isolates.

Comments and Conclusions

In this study, sequencing the 5.8S rDNA-ITS effectively separated *C. truncatum* isolates from other *Colletotrichum* species. In addition, sufficient intraspecific divergence was found among isolates of *C. truncatum* originating from different hosts, which differentiated them into several distinct strain clusters. These separations would not be possible if based only on morphological characteristics, especially between chamomile and lentil isolates of *C. truncatum*. These data support the view that distinguishable genetic differences exist between the biocontrol agent of scentless chamomile and anthracnose pathogens of lentil, pea, and soybean.

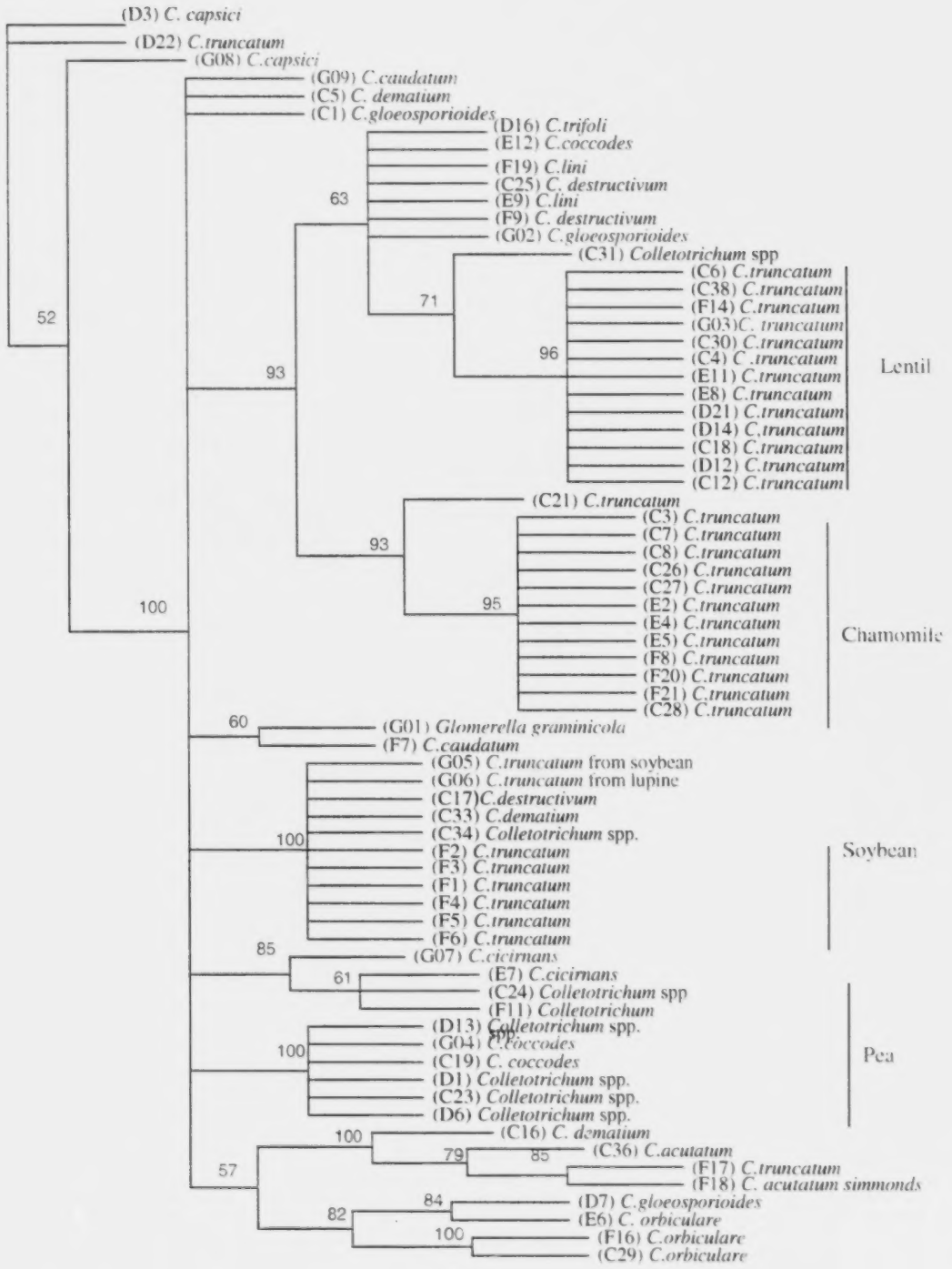

Figure VII-1. Parsimony phylogram of ITS sequences with bootstrap (>50%) generated with PAUP. Numbers in brackets are the sequence code. Sequences for the isolates in red color were obtained from the GeneBank.

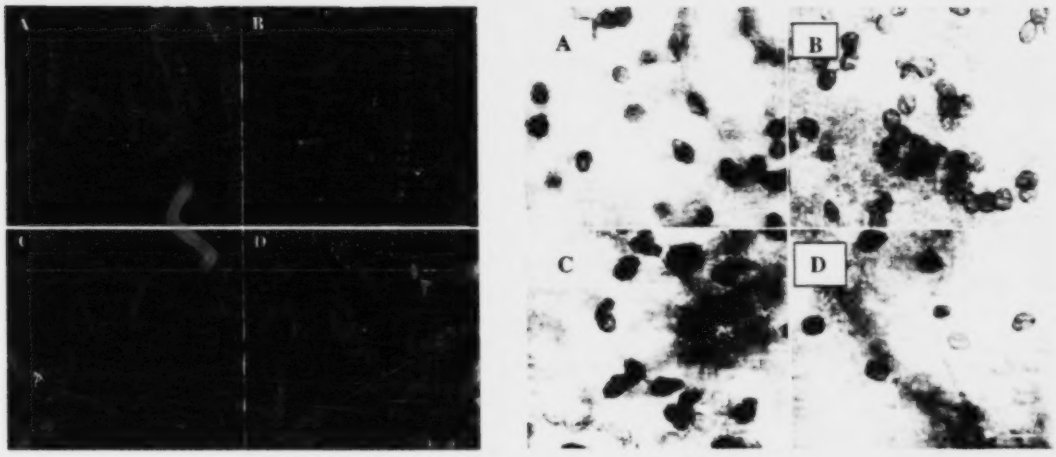

Figure VII-2. Conidia and appressoria of *Colletotrichum* spp. **a:** conidia of: lentil (A), scentless chamomile (B), soybean (C), and alfalfa (D) isolates; **b:** appressoria of: scentless chamomile (A), lentil (B), soybean (C), and pea (D) isolates. Bars = 5µm.

Table VII-1. Average size of conidia and appressoria for selected *Colletotrichum* isolates [a]

Isolate	Hosts	Size of conidia (µm) [b]	Size of appressoria (µm) [b]
00248A1	Chamomile	17.4±3.6 x 4.6±0.6	6.0±1.3 x 5.1±0.5
Ct11	Lentil	17.3±1.9 x 4.6±0.5	6.3±1.0 x 5.4±0.6
Jul0414	Pea	16.1±0.2 x 4.5±0.5	5.8±1.6 x 4.6±1.2
Jul0435	Soybean	26.4±2.1 x 3.8±1.0	10.1±0.1 x 5.0±0

[a] Based on 100 single spores and appressoria of each isolate.
[b] Length and width of conidia, and maximum and minimum diameter of appressoria.

Study VIII. Test of Ct formulations under field conditions

Background

The efficacy of biocontrol agents ought to be validated under field conditions due to potential impact by fluctuating weather variables. In addition, integrated weed management calls for use of multiple options to achieve synergy and more effective/economic weed control. While most herbicides are expected to attend a high percentage of weed kill, bioherbicides more commonly provide a level of weed suppression. With crop competition, this suppression may be of value in alleviating weed impact on crop yield and quality. Ct formulations, especially the promising tank mixtures with herbicides, were tested in field on the AAFC Research Farm near Saskatoon in 2004 and 2005.

General Methods

Each treatment plot was $1m^2$ with 25 scentless chamomile transplants. Formulations were sprayed using a compressed-air assistant sprayer fitted with an XR-8002 nozzle calibrated to deliver 200 L/ha at 275 kPa constant air pressure. Plot treatments were applied when scentless chamomile plants were at 8-10 leaf stages (2 to 3 weeks after transplanting). Applications were carried out in late afternoon or early evening and timed according to forecast overnight showers. Temperature, RH, and leaf wetness duration were monitored throughout the trial periods using a mini weather station by the plot area.

A completely randomized block design was used with 4 replicates per treatment for each trial. To assess treatment effects, plants were cut at the soil level and both fresh and dry weights were taken. Due to similar trends between the two sets of data, only fresh weight was reported as an indicator of weed control.

2004 Field Trials

Efficacy of tank-mix formulations of Ct with synergistic herbicides

Sencor at 1x label rate (0.208 kg metribuzin/ha) was tank mixed with Ct at $1x10^7$ spores/ml. Molasses or the adjuvant Score (both at 1%, v/v) was added, individually or in combination, to Ct suspensions or Ct-Sencor mixes as additional formulation ingredients. A total of three trials were conducted on plots of pure scentless chamomile stand during June and July with the following treatments included in each trial:

1. Untreated
2. Score (adjuvant along)
3. Molasses (nutrient alone)
4. Molasses plus Score
5. Sencor (herbicide alone)
6. Sencor plus Score
7. Ct alone
8. Ct plus Score
9. Ct plus Molasses
10. Ct plus Score & Molasses
11. Ct plus Sencor
12. Ct plus Sencor & Score

2005 Field Trials

Each of the following experiments was carried out twice, and the first experiments were on pure stand of scentless chamomile while the experiment 4 was in lentil plots inter-row transplanted with scentless chamomile.

Experiment 1. Ct-herbicide tank mixes for the control of scentless chamomile: Selected herbicides were used at 1x label rates as well as 0.2x and 0.1x reduced rates. The 1x label rates were 0.112 kg clopyralid plus 1.013 kg MCPA/ha for Curtail M and 0.208 kg metribuzin/ha for Sencor. The Ct dose in the tank mixes was $2x10^7$ spores/ml.

Experiment 2. Effect of Ct WOW formulation: WOW formulation was added to some of the following treatments:

1 Untreated
2 Ct in water
3 Sencor (1x)
4 Curtail M (1x)
5 WOW (formulation alone)
6 Ct + Sencor
7 Ct + Curtail M
8 Ct + WOW
9 Sencor + WOW
10 Curtail M + WOW
11 Ct + Sencor + WOW
12 Ct + Curtail M + WOW

Experiment 3. Sencor in tank mixes with variable rates of Ct: Under controlled conditions, higher Ct doses tended to provided greater suppression of scentless chamomile (Figure I-1) but a moderate Ct dose might be sufficient when tank mixed with a herbicide (Table III-2). Three Ct doses were used to tank mix with the herbicide Sencor, i.e., $1x10^7$ (Low), $3 x10^7$ (Medium) and $6x10^7$ spores/ml (High). The treatments were as the following:

1 Untreated
2 Sencor (1x)
3 Low Ct
4 Low Ct + Sencor
5 Medium Ct
6 Medium Ct + Sencor
7 High Ct
8 High Ct + Sencor

Experiment 4. Effect of Ct-Sencor tank mixtures on scentless chamomile in lentil plots: Lentil was seeded with 12-inch row spacing, and scentless chamomile plants were transplanted between rows 3 d after lentil seeding. Ct was applied at two doses ($2x10^7$ and $6x10^7$ spores/ml) in tank mixes with Sencor at 1x label rate (0.208 kg metribuzin/ha), half rate, or half rate followed with a Sencor-only (half rate) treatment a week later. At the time of treatment, lentil was at 1 to 4-node stage with a maximum height of 6 inches. Treatment effects were assessed with weights of both scentless chamomile and lentil 4 weeks after treatment. Hand-weeded plots were used as weed-free controls.

Results and Conclusions

2004 Field Trials

Efficacy of tank-mix formulations of Ct with synergistic herbicides: Overall, Ct caused only light infection on scentless chamomile due to low night temperatures during trial periods, and did not reduce fresh weight. When Ct was tank mixed with the herbicide Sencor, the treatment was more efficacious than either the component applied alone (Table VIII-1). In one of the trials where the temperature was more favourable to infection, Ct plus Sencor achieved greater weed control with noticeably higher numbers of plant kill than the herbicide alone (Appendix 1. Photo 4). The adjuvant Score or the nutrient molasses did not improve the performance of Ct, but the adjuvant with herbicide increased weed control when compared to the herbicide alone. This was possibly due to increased spray retention.

Table VIII-1. Average fresh weight (g) of scentless chamomile in 2004 field trials.

Treatment	Ct absent	Ct present
Water	24.6 abc [a]	22.3 bc [a]
Score	26.6 a	25.1 ab
Molasses	23.6 abc	21.6 c
Sencor (herbicide)	17.5 d	13.9 e
Score plus molasses	24.5 abc	23.5 bc
Sencor plus score	11.1 e	13.9 e

[a] Treatments with the same letter (s) are not significantly different ($LSD_{0.05}$).

2005 Field Trials

Experiment 1. Ct-herbicide tank mixes: Ct applied alone cause only minor infection on scentless chamomile, but the fresh weight was not affected. Both herbicides were somewhat suppressive. Curtail M alone was more effective than in 2004 but its tank mixture with Ct did not show greater efficacy. Sencor plus Ct were slightly more efficacious than the herbicide alone (data not shown).

Experiment 2. Test of Ct WOW formulation: There was a significant interaction between herbicide, Ct, and formulation, but herbicides had a much bigger impact (Table VIII-2). Ct had no significant effect possibly due to non-conducive weather conditions that limited fungal infection of the weed. However, the formulation effect was not significant when compared to Ct applied in water.

Table VIII-2. Fresh weight (g) of scentless chamomile affected by Ct formulations

Ct spore dose	No herbicide		Curtail M		Sencor	
($x10^6$ /ml)	Water	WOW	Water	WOW	Water	WOW
0	32.6 ab [b]	27.2 abc	17.2 de	16.4 de	20.4 cde	19.3 cde
20	34.4 a	27.3 abc	12.9 e	12.7 e	24.3 bcd	16.3 de

[b] Means with the same letters are not significantly different ($LSD_{0.05}$).

Experiment 3. Sencor in tank mixes with varying doses of Ct: The herbicide Sencor was the most significant factor whereas Ct alone was ineffective at all doses tested. Tank mixing Sencor with Ct increased efficacy slightly, although Ct dose alone was not a significant factor. Possibly the range of Ct doses was too narrow to show substantial differences (data not shown).

Experiment 4. Effect of Ct-Sencor tank mixtures on scentless chamomile in lentil plots: Ct alone caused only light infection on scentless chamomile but weed fresh weight or lentil biomass was not affected (Table VIII-3). Sencor alone applied in different rates or methods suppressed chamomile only slightly, but tank mixtures with Ct increased weed suppression further by 13-16% when compared to the treatments of herbicide alone. Left untreated, the scentless chamomile reduced lentil biomass by 50% when compared to the weed-free plots. Treatments with Ct plus Sencor showed less pronounced effect on lentil biomass but in split applications (0.5x herbicide each time), the combination did increase lentil biomass slightly.

Table VIII-3. Weights of scentless chamomile and lentil (g) affected by Ct plus Sencor [a]

Ct concentration	Sencor rate			
(x 10^6 spores/ml)	None	0.5x	1x	Two 0.5x split [b]
Chamomile				
0	141.3 a [c]	99.2 bc	80.2 c	83.4 c
20	119.1 ab	87.5 bc	63.1 de	NA
60	119.0 ab	89.2 bc	69.5 d	70.3 d
Lentil biomass				
0	33.0 A	42.9 BC	44.1 C	40.4 B
20	34.6 A	38.5 B	36.5 AB	NA
60	36.1 AB	39.8 B	45.8 C	46.6 C

[a] Dry weight of lentil was taken for biomass, and the average for weed-free plots was 65.9 g.
[b] Two applications of Sencor at 0.5x rate each time. The 2nd application was Sencor only.
[c] Means with the same letters and on the same plant are not significantly different ($LSD_{0.05}$).

Comments and Conclusions

Consistent performance of biocontrol agents under field conditions prove to be challenging. For foliar applied organisms, the biggest hurdle is to overcome the impact of fluctuating weather variables. In the prairies, both temperature and moisture can be limiting. In general, Ct applied alone did not achieve weed suppression probably due to cooler temperatures experienced during trial periods (often below 10°C at night). Tank mixing Ct with the herbicide Sencor or Curtail M appeared more promising and the fungus was able to take advantage of weed predisposition by the herbicides and light infection often progressed into more severe forms of damage. Yield-reduction potential by scentless chamomile infestation was shown in lentil plots and timely control of the weed is of great economic importance. The less pronounced effect of the tank-mix treatment on lentil biomass is probably due to a merely slight increase in weed control than the herbicide alone and a too short duration of weed/crop competition (4 weeks after treatment). It is clear that the microbial formulation will have to be improved further for a bigger impact on field performance of this biocontrol agent.

Final Remarks

This project investigated potential use of Ct with herbicides for synergy, mass production using submerged fermentation, formulations for efficient field delivery and performance, and potential risks of Ct to important field crops in the prairies. The benefit of combining Ct with selected herbicides was clearly demonstrated, especially with Sencor. This tank mix provided an option for the control of scentless chamomile in lentil crops. Significant progress was made for Ct production using submerged fermentation, with critical nutritional and environmental factors identified. Further development of scale-up protocols will ensure capturing the value of earlier investment into this project. Formulation additives that enhanced spray retention or stimulated fungal germination showed incremental enhancement of efficacy under controlled conditions, but these improvements were insufficient to make substantial differences in field performance of the biocontrol agent. Continued formulation efforts should be placed on overcoming the impact of fluctuating weather variables under the semi-arid prairie climate. DNA sequence characteristics obtained from the molecular study will not only differentiate the biocontrol agent from anthracnose pathogens on lentil and pea, but also generate unique molecular markers for various *C. truncatum* strains that can be used further for a range of research purposes such as studying environmental fate of the biocontrol agent after release and identifying anthracnose pathogens from crop plants.

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Publications and Presentations

Scientific publications:

1. Graham G.L., Peng, G., K.L. Bailey, and F.A. Holm. 2006. Effect of plant leaf stage and fungal inoculum dose on control *Matricaria perforata* with *Colletotrichum* sp. plus herbicides. BioControl 50 (accepted).
2. Graham G.L., Peng, G., K.L. Bailey, and F.A. Holm. 2006. Interactions of *Colletotrichum* sp. with herbicides for control of scentless chamomile (*Matricaria perforata*). Weed Technol. 15 (in press).
3. Graham G.L., Peng, G., K.L. Bailey, and F.A. Holm. 2005. Effect of dew temperature, post-inoculation condition, and pathogen dose on suppression of scentless chamomile by *Colletotrichum truncatum*. Biocontrol Science & Technology 16:271-280 (attached).

Conference proceedings:

1. Graham, G.L.,G. Peng, K.L. Bailey and F.A. Holm. 2003. Preliminary study on synergy of herbicides and microbial agents for improved control of scentless chamomile. Page 82-86 in Proceedings of 56th Canadian Weed Science Society Annual Meeting in Saskatoon, SK.
2. Peng, G. and G.L. Graham. 2003. Control of scentless chamomile using a host-specific fungus and fungus/herbicide synergy. Page 5-8 *In:* Proc. of 2003 Saskatchewan Alfalfa Seed Conference.
3. Dokken, F.L., G. Peng, L.M. Nelson, and K.L. Bailey. 2004. Submerged fermentation of *Colletotrichum truncatum* for biological control of scentless chamomile. In: Proc. of Soil and Crop Workshop, University of Saskatoon (4 pp in CD). February, 2004, Saskatoon, SK, Canada.

Conference presentations and posters:

1. Graham, G.L., G. Peng, K.L. Bailey, and R. Holm. 2003. Effect of dew temperature, post-inoculation condition and pathogen concentration on infection and disease caused by *Colletotrichum truncatum* on scentless chamomile. Presentation at Can. Phytopath. Soc. & Sask. Regional Group & Phytopath. Soc. of Alberta joint annual meeting. Abstract in Can. J. Plant Pathol. 26:225.
2. Dokken, F.L. G. Peng, K.L. Bailey, and L.M. Nelson. 2003. Submerged fermentation of *Colletotrichum sp.* for biological control of scentless chamomile. Poster at Can. Phytopath. Soc., Saskatchewan Regional Group & Phytopath. Soc.of Alberta joint annual meeting. Abstract in Can. J. Plant Pathol. 26:224 (poster attached).
3. Peng, G. 2003. Control of scentless chamomile with an integrated approach using plant pathogens with herbicides. Presentation at Saskatchewan Alfalfa Seed Growers Assoc. Annual Meeting: Jan. 9, 2003.

4. Peng, G. and G.L. Graham. 2003. Potential for improved control scentless chamomile using fungal pathogens and registered herbicide. Presentation at Saskatchewan Scentless Chamomile Working Group Annual Meeting, Nov. 17, 2003.

5. Dokken, F.L., G. Peng, L.M. Nelson, and K.L. Bailey. 2004. Submerged fermentation of *Colletotrichum* sp. for biocontrol of scentless chamomile. Presentation at Soil & Crop Workshop sponsored by U of S. February 2004, Saskatoon, SK.

6. Peng, G. 2004. Strategies for enhancing efficacy of bioherbicide agents. Presentation at the symposium of Canadian Forum for Biological Control annual meeting – Biological Control: From Discovery to Application. June 13, 2004, Ottawa.

7. Peng, G. 2004. Control of scentless chamomile using an integrated approach. Presentation at Saskatchewan & Manitoba Industrial Vegetation Management Annual Meeting. Sept.17, 2004, Regina.

8. Peng, G. 2004. Development of a bioherbicide strategy for control of scentless chamomile – How it fared under field conditions. Presentation at Saskatchewan Scentless Chamomile Working Group Annual Meeting, December 6, 2004, Saskatoon,

9. Liu, L, G. Peng, Y.D. Wei, B.D. Gossen, and K.L. Bailey. 2005. Differentiation of *Colletotrichum* spp. from scentless chamomile and lentil based on infection characteristics and ITS sequences. Poster presentation at Canadian Phytopathological Society annual meeting. Edmonton. Can. J. Plant Pathol. 26: 472 (abstr.).

10. Russell K. Hynes, Paulos Chumala, Daniel Hupka and Gary Peng. 2005. W/O/W emulsions - Formulation development for foliar application of bioherbicides. Poster at Canadian Weed Science Society annual meeting. Niagara falls.

11. Forseille, L.,Wei, Y.D. and Peng,G. 2005. Differentiation and detection of *Colletotrichum truncatum* isolates by strain-specific PCR markers. Presentations at the annual meeting for the Saskatchewan Group of Canadian Phytopathological Society, Saskatoon. Can. J. Plant Pathol.27 (abstr. in press)

12. Faye L. Dokken, Gary Peng, M. Rollins, and A. Friesen. 2006. Potential of submerged fermentation for mass production of *Colletotrichum truncatum*, a mycoherbicide agent against scentless chamomile. Poster at 5th Annual Meeting of Biocontrol Network (poster attached).

Personnel (salaries/wages were paid out of this project fund)

Gavin Graham, Research Affiliate (M.Sc. student) and Term Technician (1 year)

Faye Dokken, Research Affiliate (M.Sc. student)

Lily Forseille, Research Affiliate (M.Sc. student)

Equipment

No equipment or supply over $1000 was purchased using the project fund.

Project Developed Materials:

1. A group of host-specific *Colletotrichum truncatum* isolates has been identified as candidates for biocontrol of scentless chamomile. Pathological and epidemiological requirements by these isolates have been determined in relation to typical weather conditions under which they will be applied.
2. Two herbicides have been demonstrated to be synergistic with the biocontrol agent in tank-mix applications for increased weed control.
3. A liquid medium has been developed for potential mass production of fungal spores using submerged fermentation.
4. PCR-specific markers have been developed and can be used effectively for detection and differentiation of *Colletotrichum* strains from scentless chamomile, lentil, pea, and soybean. These markers have broader applications in tracking the biocontrol agent after release and correctly identifying the causal agent of anthracnose on pulse crops.

Project Photos

Four illustration photos are included in Appendix 1.

Acknowledgement

Monetary contributions by ADF of Saskatchewan Department of Agriculture, Food, and Rural Revitalization has been acknowledged in all publications, presentations, and posters.

Expense Statement

A financial statement is included in Appendix 2. With the instalment of the fund, the account should be balanced.

Appendix 1. Project Photos

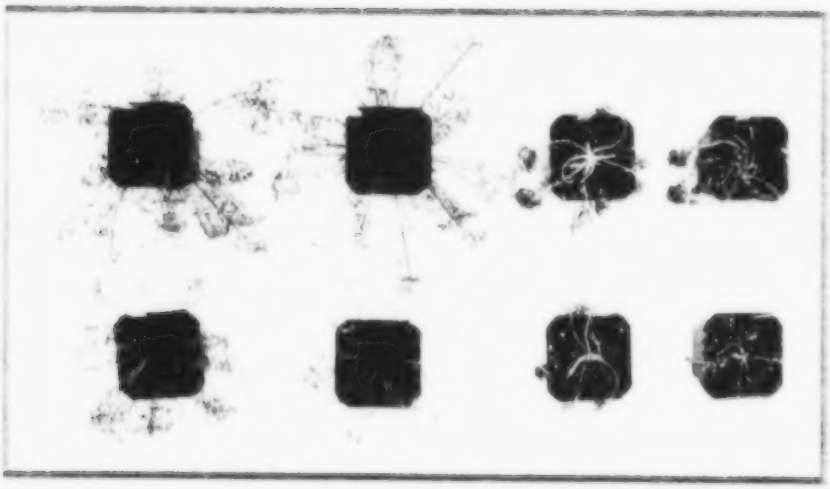

Photo 1. Effect of Curtain M plus *Colletotrichum truncatum* (Ct) on scentless chamomile at 8-leaf (lower row) and 11-leaf stages. From left to right: controls, Ct alone, Curtail M, and Curtail M plus fungus. Weed kill was achieved at the 8-leaf stage only (circled plant).

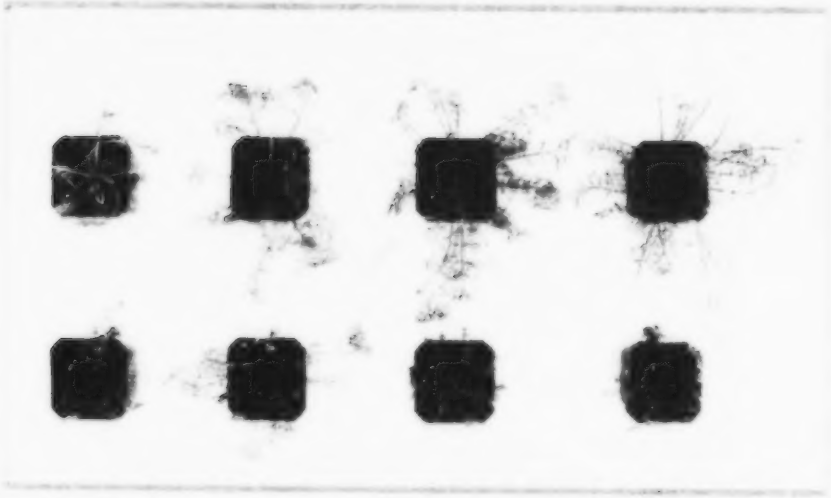

Photo 2. Control of scentless chamomile at the 11-leaf stage by Sencor alone (upper row) and Sencor plus *Colletotrichum truncatum* in a tank mix (lower row).

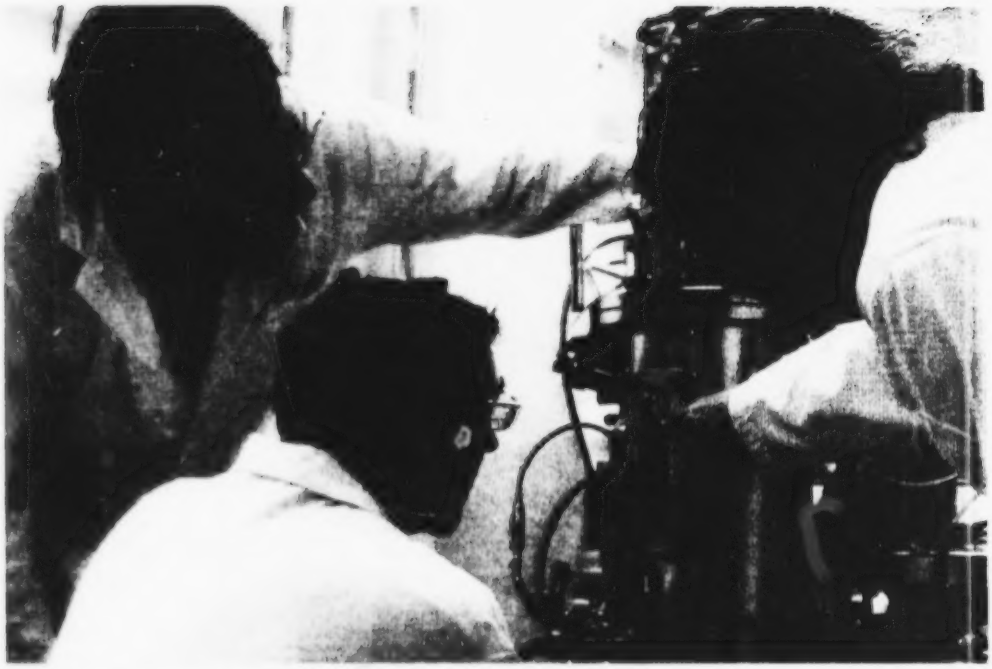

Photo 3. Production of *Colletotrichum truncatum* spores using bench-top fermentors at the Fermentation Pilot Plant, Saskatchewan Research Council.

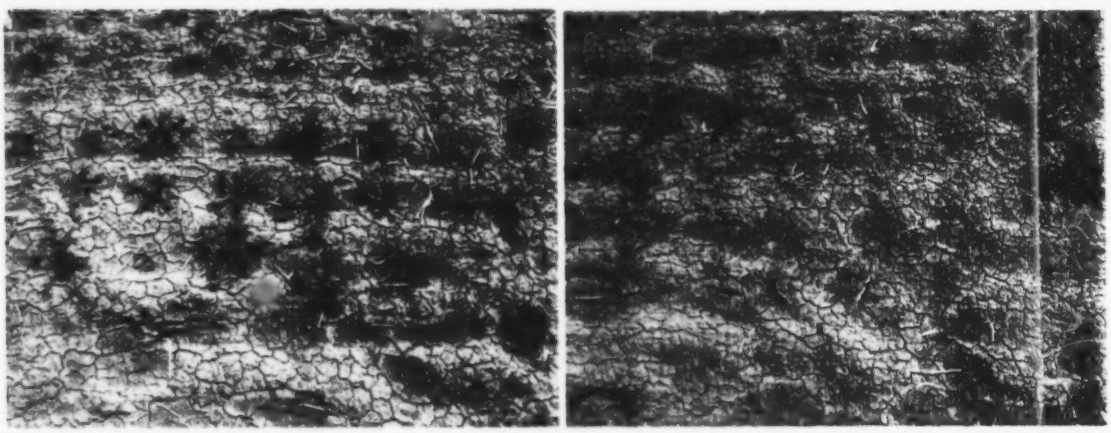

Photo 4. Control of scentless chamomile under field conditions with Sencor (picture on the left) and Sencor plus *Colletotrichum truncatum* in a tank mix (picture on the right). The tank mix caused higher percentage of weed kill than did the herbicide alone.